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14. ABSTRACT
The traditional therapeutic and surgical approaches have not been successful in the management of prostate cancer (CaP). Natural plant-based products have shown promise as anticancer agents. Sanguinarine, a benzophenanthridine alkaloid derived from the root of Sanguinaria Canadensis, has been shown to possess anti-microbial, antioxidant and anti-inflammatory properties. Our earlier studies suggested that sanguinarine may be developed as an agent for the management of prostate cancer. Based on this rationale, funded by the DOD (Award - W81XWH-04-1-0220), we initiated a study to investigate the hypothesis that sanguinarine will impart antiproliferative effects against prostate cancer via a modulation in NF-kB-pathway-mediated apoptosis. In the last three years, we have made reasonable progress towards our goals. However, the progress during this reporting period was hampered due to several unforeseen circumstances. Because of this reason, a one-year extension of the grant was obtained in January 2007. So far, the key accomplishments of our project are as follows. We have shown that sanguinarine possesses chemopreventive/anti-proliferative effects against CaP in an athymic nude mice xenograft model. Further, our data also suggested that sanguinarine-caused effects are mediated via modulations in NF-kB pathway and cki-cyclin-cdk machinery. At present, the studies are ongoing to assess the chemopreventive/therapeutic effects of sanguinarine on CaP development in transgenic TRAMP model.

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Introduction:

In American men, **Ca**ncer of the **P**rostate (**CaP**), continues to be one of the most frequently occurring malignancies, representing ~29% of all new cancer cases (1). The traditional surgery and therapy has not been successful in the management of CaP. Therefore, the search for novel agents and approaches for the treatment of CaP continues. Natural plant-based products have shown promise as anticancer agents. Ideally, the anti-cancer drugs should specifically target the neoplastic cells with minimal "collateral damage" to normal cells. Thus, the agents, which can eliminate the cancerous cells without affecting the normal cells, may have therapeutic advantage for the elimination of cancer cells. Sanguinarine (13-methyl[1,3]benzodioxolo[5,6-c]-1,3-dioxolo[4,5-i]phenanthridinium), derived from the root of *Sanguinaria Canadensis* and other poppy-fumaria species, is a benzophenanthridine alkaloid and a structural homologue of chelerythrine and has been shown to possess anti-microbial antioxidant and anti-inflammatory properties (2-4). Our published and preliminary studies have suggested that sanguinarine may be developed as an agent for the management of prostate cancer (5-6). Based on this rationale, funded by the Department of Defense (DOD; Idea Development Award - W81XWH-04-1-0220), we initiated a study to investigate the hypothesis that *sanguinarine will impart antiproliferative effects against prostate cancer via a modulation in NF-κB-pathway-mediated apoptosis*. Overall, we have made significant progress towards achieving our goals.

Based on our preliminary studies and the progress so far, we expect that a successful completion of this proposal will define i) the potential of sanguinarine against CaP, and ii) molecular mechanism(s) of the biological effects of sanguinarine. This may pave the way for the development of novel strategies for the management of CaP.

Main Body of the Progress Report:

In the 'Idea Development Award' selected for funding by the 'US Army Medical Research and Material Command', we proposed to test the hypothesis that ***a plant-derived alkaloid sanguinarine will impart antiproliferative effects against prostate cancer via a modulation in NF-κB-pathway-mediated apoptosis***.

Overall, we have made considerable progress with respect to our proposed hypothesis. However, unfortunately, our progress during this reporting period (01/16/2006 - 01/15/2007) was hampered due to several unforeseen circumstances. Because of this reason, a one-year no-cost extension of the grant was obtained in January 2007. **Our experiment with TRAMP mice (from our breeding colony) could not be completed. Because of unexplainable reasons, the TRAMP mice died during the ongoing experiment to assess the chemopreventive effects of sanguinarine against CaP. Following this mishap, we tried to obtain the TRAMP mice from Jackson laboratory which was not able to supply the required number of mice. So, we established an agreement with the Jackson laboratory according to which we will continue to obtain 32 mice every three months. We have obtained the first batch of mice and started our proposed studies. We are extremely hopeful that we will be able to complete the work proposed during this extension.**

A brief description of the progress made in last two years is presented in the following pages.

<u>Evaluation of anti-proliferative effects of sanguinarine against prostate cancer in nude mice xenografts.</u>

Continuing with our ongoing work, we conducted detailed studies to determine the efficacy of sanguinarine against prostate cancer in athymic nude mice implanted with human prostate cancer cells. Further, we conducted experiments to determine the molecular mechanism associated with the observed anti-proliferative effects of sanguinarine. A brief account of this study is given below.

Study Design:

To determine the chemopreventive and therapeutic potential of sanguinarine against CaP in vivo, we employed the athymic nude mice xenografts model. For this experiment, the athymic (nu/nu) male nude mice (obtained from NxGen Biosciences, San Diego, CA) were randomly divided into different groups of 10 animals each and CW22Rv1 cells ($1x10^6$ cells in 50 µl RPMI + 50 µl Matrigel) were implanted in athymic nude mice by a sub-cutaneous injection on left and right sides, below the shoulders (2 tumors/mouse). The rationale for the choice of CWR22Rv1 cells is based on the fact that our major goal was to determine the chemopreventive effects of sanguinarine in early stages of CaP development, when the disease is androgen-dependent. Another reason for employing CWR22Rv1 cells was that they make PSA, which is arguably considered a *gold standard* for monitoring the CaP in humans. The animals were treated with sanguinarine (1 or 5 mg/kg body weight in 0.2 ml PBS, five days a week) by intra-peritoneal injection either one week post cell implantation to establish the preventive potential or after the development of a sizable tumor (200 mm^3) to examine the therapeutic potential. Thus, two different protocols were employed.

The detail of **Protocol-1** is as follows:

Group I: Control – cells were implanted (at the start of the experiment; day 0), no further treatment given;

Group II: Sanguinarine (1 mg/kg) – cells were implanted on day 0 and the mice were injected with sanguinarine (1mg/kg body weight; *i. p.*) 5 days/ week (Monday – Friday);

Group III: Sanguinarine (5 mg/kg) – cells were implanted on day 0 and the mice were injected with sanguinarine (5mg/kg body weight; *i. p.*) 5 days/ week (Monday – Friday)

The detail of **Protocol-2** is as follows:

Group 1: Control – cells were implanted (at the start of the experiment), no further treatment was given;

Group 2: Sanguinarine (1 mg/kg) – cells were implanted and the tumors were allowed to grow and achieve a volume of 200 mm^3, when the treatment with sanguinarine (1mg/kg body weight; *i. p.*; 5 days/ week – Monday – Friday) was started and continued until the termination of experiment;

Group 3: Sanguinarine (5 mg/kg) – cells were implanted and the tumors were allowed to grow and achieve a volume of 200 mm^3, when the treatment with sanguinarine (5mg/kg body weight; *i. p.*; 5 days/ week – Monday – Friday) was started and continued until the termination of experiment;

In both the protocols, the control animals received vehicle only. The treatment schedule was continued until the tumors reached a volume of 1000 mm^3. At this point, the animals were withdrawn from the study and euthanized. Throughout the experiment the animals were housed under standard housing conditions and had free access to autoclaved laboratory chow diet. In this protocol, to assess the possibility of treatment-toxicity, the effect of treatments on food/water consumption and body weight was monitored twice weekly throughout the study. Further, blood was withdrawn periodically to determine the effects of treatments on PSA levels in serum. The effect of sanguinarine treatment was determined on the growth of implanted tumors and the serum levels of prostate specific antigen (PSA).

At the termination of experiment, the tumors were harvested for further studies to evaluate the mechanism of the antiproliferative effects of sanguinarine against prostate cancer.

Results & Conclusion:

Anti-proliferative Effects of Sanguinarine Against CaP: As shown in on the next page (Figures 1 & 2), our data demonstrated that sanguinarine (both pre- and post- treatments) resulted in a highly significant inhibition in the rate of tumor growth as assessed by a regression analysis. Further, the Kaplan-Meier Analysis demonstrated that in sanguinarine treated animals (post-treatment), the rate of tumor growth (to reach to a 1000 mm3 target volume) was significantly delayed. Furthermore, treatment of mice with sanguinarine (both pre- and post- tumor) resulted in a significant reduction in serum levels of prostate-specific antigen (PSA) in nude mice implanted with CWR22Rv1 cells.

A) Sanguinarine pre-treatment

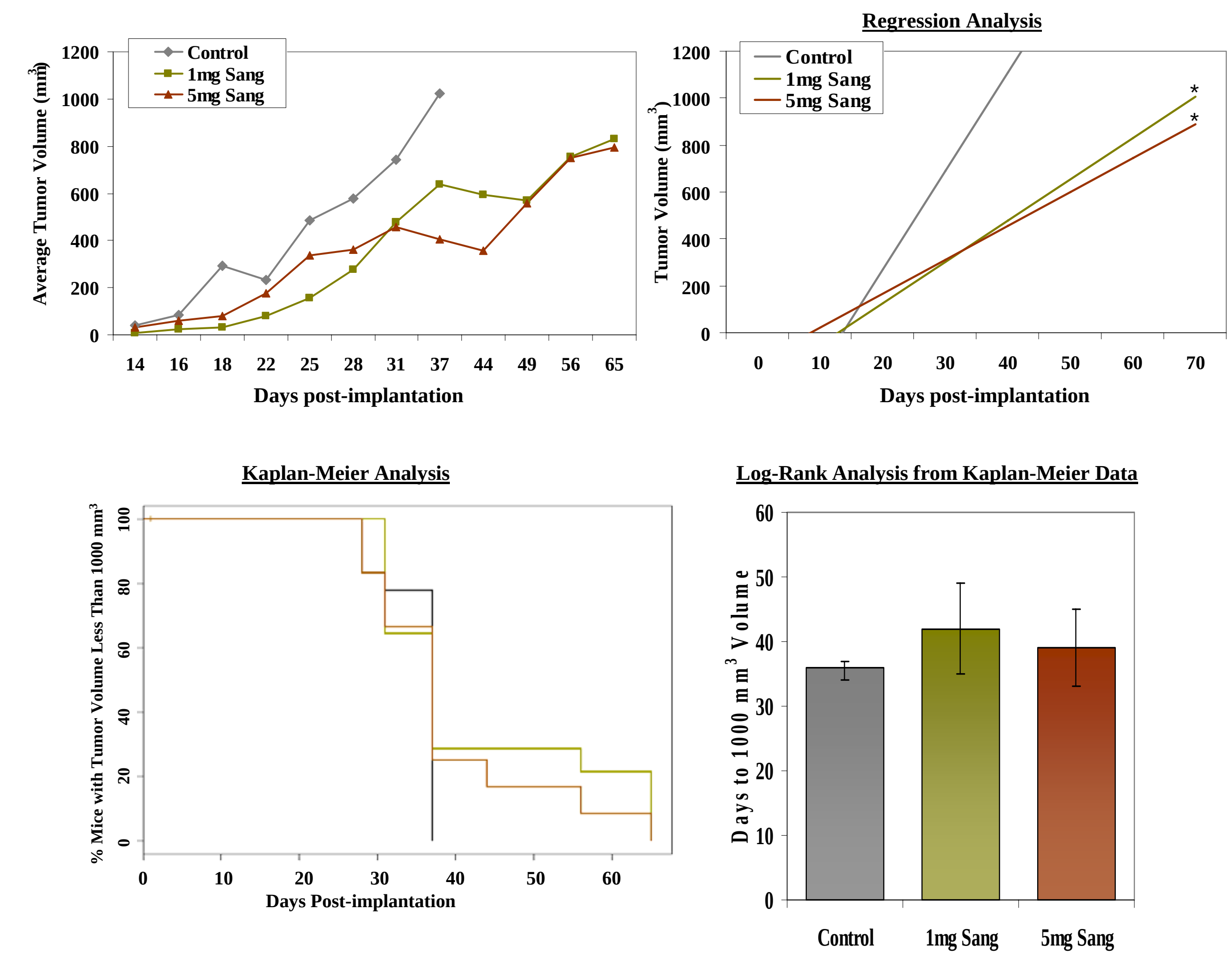

Fig. 1: Effects of sanguinarine treatment on the growth of CWR22Rv1 cell- implanted prostate tumors in athymic nude mice. The cells (in matrigel) were implanted on both flanks of nude mice. The animals were treated with sanguinarine as described above. The effect of sanguinarine pre-treatment was measured in terms of average tumor volume of as a function of time. Further, the rate of tumor growth was assessed by linear regression analysis. Tumor-free survival was assessed by Kaplan-Meier plot and the average time to reach 1000 mm^3 tumor volumes was assessed by Log-Rank analysis of Kaplan-Meier data. *p<0.05 was considered significant.

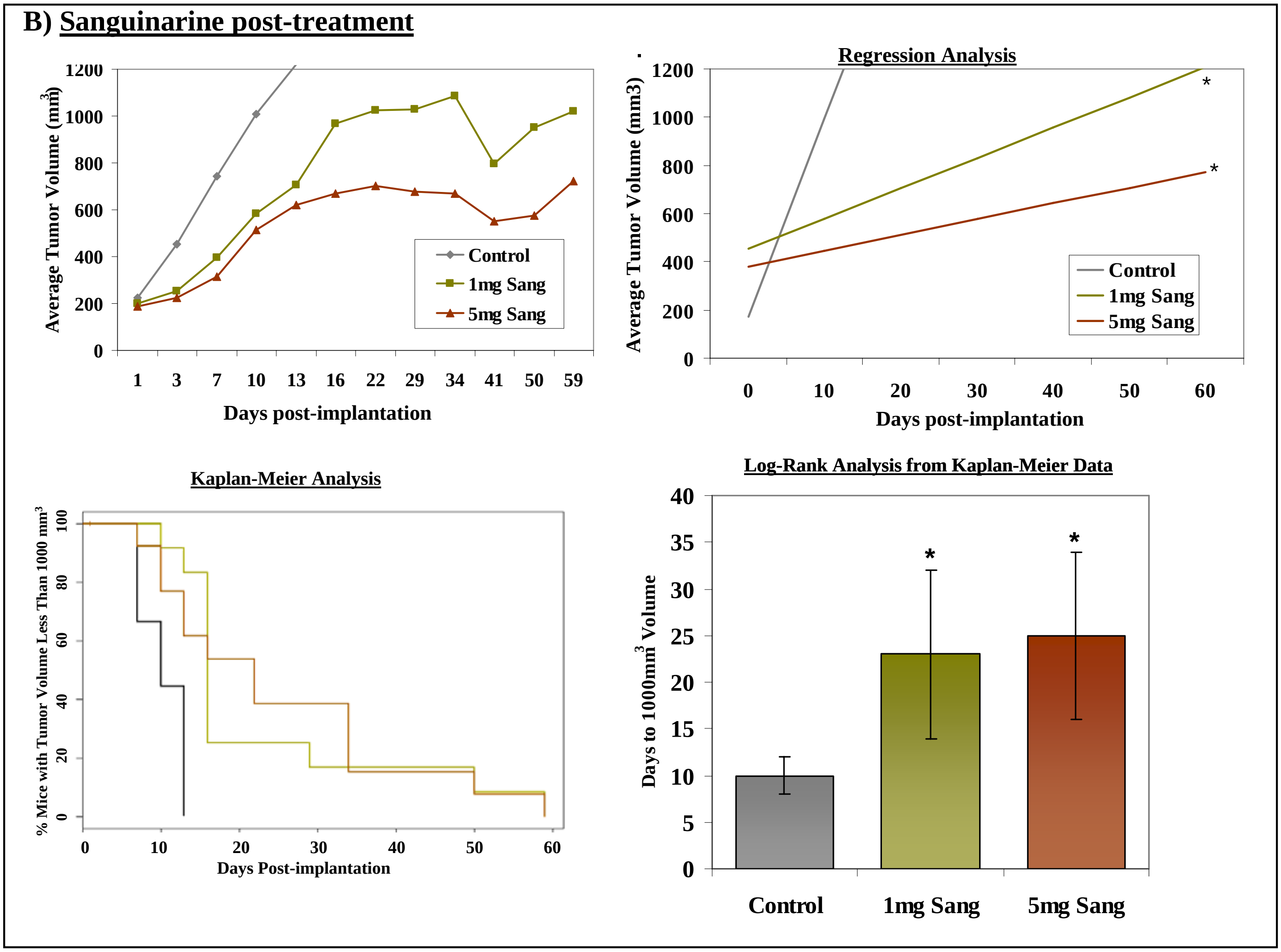

Fig. 2: Effects of sanguinarine post-treatment on the growth of CWR22Rν1 cell- implanted prostate tumors in athymic nude mice. The cells (in matrigel) were implanted on both flanks of nude mice. The animals were treated with sanguinarine as described above. The effect of sanguinarine treatment on the growth of established tumors was measured in terms of average tumor volume of as a function of time. Further, the rate of tumor growth was assessed by linear regression analysis. Tumor-free survival was assessed by Kaplan-Meier plot and the average time to reach 1000 mm^3 tumor volumes was assessed by Log-Rank analysis of Kaplan-Meier data. *$p<0.05$ was considered significant.

Effect of Sanguinarine on Serum PSA: Further, as described earlier, for this study, we used CWR22Rν1 cell because these cells are known to secrete PSA. As shown in figure 3, our data clearly demonstrated that treatment of mice with sanguinarine (both pre- and post- tumor) resulted in an appreciable reduction in serum levels of prostate-specific antigen (PSA) in nude mice implanted with CWR22Rν1 cells. This is an important observation because serum PSA is considered to be an important marker for identifying humans CaP and, several investigators have also reported the usefulness of serum PSA as a follow up marker for local recurrence and/or distant disease in the patients after radical prostatectomy, radiation and hormonal therapy.

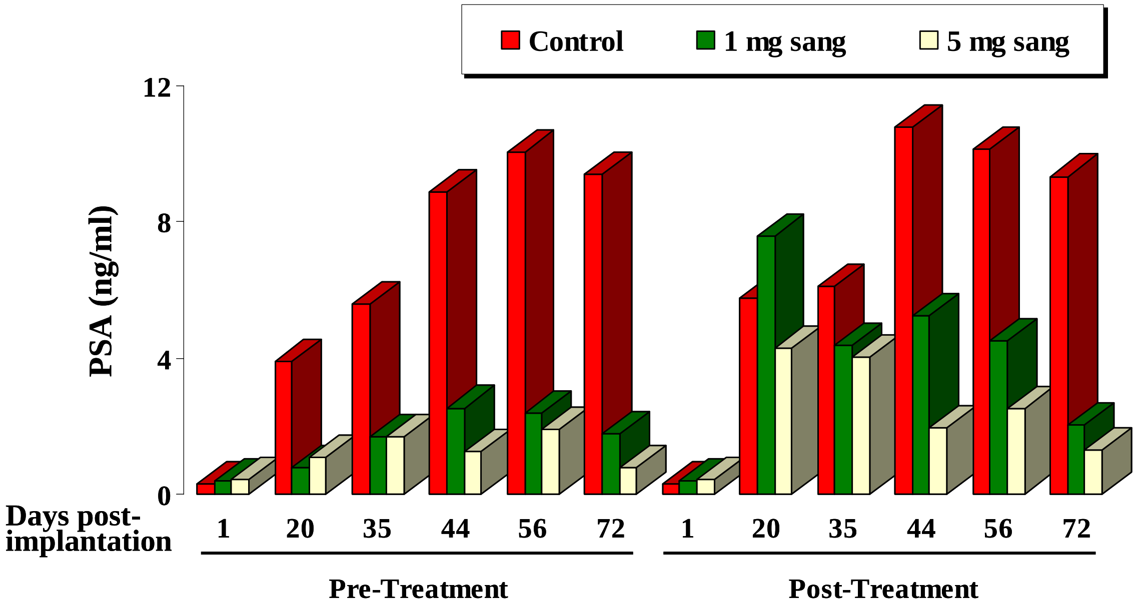

Fig. 3: Effects of sanguinarine treatment on the levels of serum PSA in athymic nude mice implanted with CWR22Rν1 cells. The cells (in matrigel) were implanted on both flanks of nude mice. The animals were treated with sanguinarine as described above. For determining PSA levels in the serum, following the treatments of animals with sanguinarine, at different times post-ctumor cell inoculation, blood was collected by 'madibular bleed' and serum was separated. The levels of PSA were determined by using a quantitative Human PSA enzyme linked immunosorbent assay (ELISA) kit (Anogen, Ontario, Canada) as per the manufacturer's protocol.

Thus, this study, for the first time, demonstrated the chemopreventive and therapeutic effects of sanguinarine against PCa development under in vivo situations. Based on our data, we suggest that sanguinarine is a promising candidate for chemoprevention and/or intervention against PCa.

<u>Effect of Sanguinarine on NF-κB Pathway:</u> Further, we also determined the mechanism of growth inhibitory effects of sanguinarine against CaP in nude mice implanted with CWR22Rν1 tumors. As shown below in figure 4, we found that sanguinarine treatments (in both the protocols) resulted in an appreciable down-modulation in the protein levels of NF-κB/p65 (in the nucleus) suggesting that the observed effects of sanguinarine may be mediated via inhibition of NF-κB pathway.

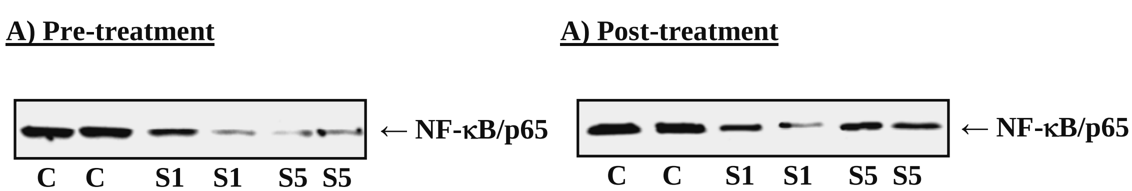

Fig. 4: Effect of sanguinarine on nuclear levels of NF-κB/p65 in nude mice implanted with CWR22Rν1 tumors. The cells (in matrigel) were implanted on both flanks of nude mice. The animals were treated with sanguinarine as described above. At the end of experiment, the mice were sacrificed and tumors were surgically removed and the levels of nuclear NF-κB/p65 were examined by the western blot analysis using the appropriate primary and secondary antibodies. The data shown here are representative of three independent immunoblots with similar results. **C = control; S1 = sanguinarine 1mg/ml; S5 = sanguinarine 5mg/ml.**

In addition, as shown below in figure 5, our data also demonstrated that sanguinarine treatment resulted in an appreciable decrease in the protein levels of anti-apoptotic Bcl-2 that is an established downstream target of NF-κB.

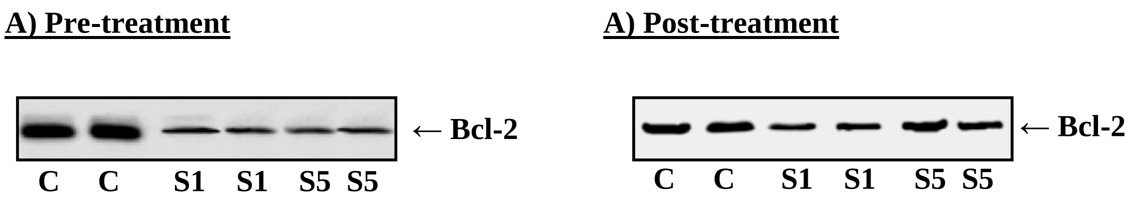

Fig. 5: Effect of sanguinarine on Bcl-2 protein levels in nude mice implanted with CWR22Rν1 tumors. The cells (in matrigel) were implanted on both flanks of nude mice. The animals were treated with sanguinarine as described above. At the end of experiment, the mice were sacrificed and tumors were surgically removed and the levels of Bcl-2 proteins were examined in total cell lysate with western blot analysis using the appropriate primary and secondary antibodies. The data shown here are representative of three independent immunoblots with similar results. **C = control; S1 = sanguinarine 1mg/ml; S5 = sanguinarine 5mg/ml.**

Further, as shown in figure 6, our data also demonstrated that a down-modulation of cyclin D1 and cdk2 and an upregulation of WAF1/p21 during sanguinarine-mediated growth inhibition of CWR22Rν1-implanted prostate tumors in nude mice.

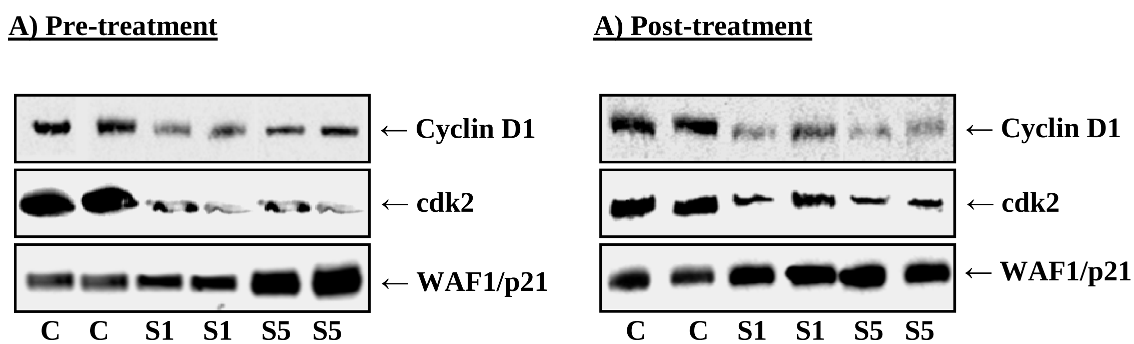

Fig. 6: Effect of sanguinarine on cell cycle regulatory proteins in nude mice implanted with CWR22Rν1 tumors. The cells (in matrigel) were implanted on both flanks of nude mice. The animals were treated with sanguinarine as described above. At the end of experiment, the mice were sacrificed and tumors were surgically removed and the levels of proteins were examined in total cell lysate with western blot analysis using the appropriate primary and secondary antibodies. The data shown here are representative of three independent immunoblots with similar results. **C = control; S1 = sanguinarine 1mg/ml; S5 = sanguinarine 5mg/ml.**

These studies are currently ongoing; and we are performing immunohistochemical- Real-Time PCR- and Western Blot- analyses to unravel the effect of sanguinarine on NF-κB pathway, Bcl-2 family protein and *cyclin kinase inhibitor-cyclin-cyclin dependent kinase network* in athymic nude mice implanted with CWR22Rν1 tumors.

Evaluation of anti-proliferative effects of sanguinarine against prostate cancer in transgenic adenocarcinoma mouse prostate (TRAMP) model.

Study Design:

In order to assess the feasibility of our plan to determine the chemopreventive/therapeutic effects of sanguinarine against CaP in transgenic TRAMP mice that mimic the features of human disease, we first conducted a preliminary experiment with limited number of animals. In our preliminary studies, 12 male hetrerozygous C57BL/TGN TRAMP mice, line PB Tag 8247NG (12-14 weeks old; obtained from our breeding colony at the Animal Care Facility, School of Medicine, University of Wisconsin) were divided into three groups and subjected to sanguinarine treatments as indicated below:

Group 1: Control (PBS alone; *intraperitonial injection*; 5 days/week)
Group 2: Sanguinarine (1 mg/kg in PBS; *intraperitonial injection*; 5 days/week)
Group 3: Sanguinarine (5 mg/kg in PBS; *intraperitonial injection*; 5 days/week)

This treatment was given to the animals 5 days per week for 6 weeks. Throughout each experiment, the animals had free access to laboratory chow and water *ad libitum*. At the end of six weeks, the experiment was terminated and the animals from both experimental and control groups were sacrificed. At the time of sacrifice, the lower GU tract, including the bladder, testes, seminal vesicles, and prostate, was removed en bloc. The GU wet weight was recorded followed by surgical dissection of the prostate gland. The weight of whole prostate gland was also recorded. Protein lysates were prepared from the prostate glands by homogenization in lysis buffer followed by the immunoblot analysis.

Results & Conclusion:

As shown in figure 7, we found that compared to control, sanguinarine treatments resulted in an appreciable decrease in GU weight and prostate weight. Further, the treatments were not found to have any evident toxic effects (body weight, food/fluid consumption) on the TRAMP mice.

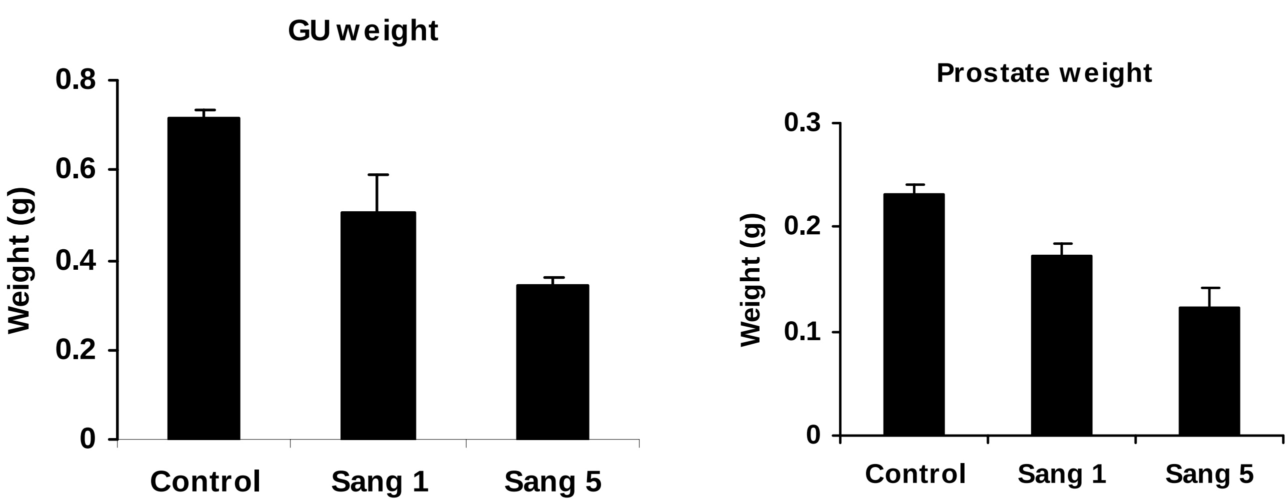

Fig. 7: Effect of Sanguinarine on GU and prostate weights in TRAMP mice. The mice were treated with sanguinarine as described above. At the end of experiment, the mice were sacrificed and GU and prostate weights were recorded. The data showed here represent mean ± SEM from four animals per group.

Further, we also assessed whether or not sanguinarine treatment affects the levels of transgene in TRAMP mice. As shown by the immunoblot analysis (Figure 8), treatment of mice with sanguinarine did not result in any significant change in the levels of transgene protein expression. This data clearly suggested that TRAMP mice are suitable model for our proposed pre-clinical trial.

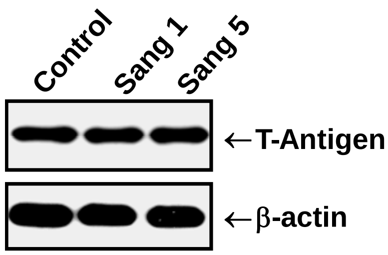

Fig. 8: Effect of sanguinarine on protein levels of T-antigen in the prostate of TRAMP mice. The effect of sanguinarine on the protein levels of T-antigen was assessed by immunoblot analyses using anti SV40TagAg antibody obtained from Santa Cruz Biotechnology., Inc. Equal loading was confirmed by reprobing the blot with β-actin. The data shown here are representative of three independent immunoblots with similar results.

However, these are very preliminary short-term studies conducted with very limited number of animals. Thus, while these studies provide a rationale and support our working hypothesis, any conclusion can not be drawn from these data.

Future Plan:

We are conducting additional experiments needed and proposed under specific aim 3. A brief description of the proposed aim is as follows:

We will assess the chemotherapeutic effects of sanguinarine on prostate cancer development and its metastasis under *in vivo* situation employing transgenic adenocarcinoma mouse prostate (TRAMP) mice, which spontaneously develop metastatic-CaP that closely mimics human disease. This specific aim is designed to study the therapeutic effect of sanguinarine against prostate cancer under *in vivo* situations in an animal model that closely mimics human prostatic disease and therefore possesses high relevance to human CaP. The major advantage of this model is that metastatic CaP, in these animals, developes spontaneously without any chemical or hormonal treatment. This specific aim has two goals.

A. **We will assess the effect of sanguinarine on CaP development and its subsequent metastasis.** The effect on CaP development will be monitored by analyzing tumor growth by a recently perfected technique of magnetic resonance imaging (MRI) and tumor weight, volume and characteristics. The effect of sanguinarine on metastasis will be ascertained by classical 'India ink method', histology and by following the markers of metastasis *viz*. E-cadherin, α- and β- catenins and integrins (α6β1 and α6β4). In addition, we will also evaluate the effect of sanguinarine on probasin to confirm whether or not it affects the statement of promoter.

B. **We will assess the involvement of NF-κB-pathway as a mechanism of antiproliferative effects of sanguinarine *in vivo* during inhibition of CaP development in TRAMP mice.** We will evaluate the effect of sanguinarine on i) induction of apoptosis (DNA fragmentation, PARP cleavage), ii) cellular proliferation (PCNA and Ki-67 expression), and iii) modulations in NF-κB-pathway (levels of NF-κB, IκB, IKK, NIK), during sanguinarine-mediated inhibition of CaP tumorigenesis in TRAMP mice. We will also evaluate the effect of sanguinarine on the modulation of NF-κB-regulated gene products: i) anti-apoptotic proteins Bcl-2 and Bcl-xL, and ii) activation of pro-apoptotic procaspase-3 and procaspase-8.

Key Research Accomplishments:

Based on our progress in the last two years of funding, the key research accomplishments are itemized below.

1. *We have demonstrated that sanguinarine possesses chemopreventive/anti-proliferative effects against prostate cancer in an athymic nude mice xenograft model. Further, our data suggested that sanguinarine-caused effects are mediated via modulations in NF-κB pathway and cyclin kinase inhibitor-cyclin-cyclin dependent kinase machinery.*

2. *Our pilot study has shown that transgenic TRAMP model, which mimics human disease, is suitable for chemoprevention/intervention studies with sanguinarine*

Reportable Outcome:

The following publications are associated with the funding from the DOD.

1. Adhami VM, Aziz MH, Reagan-Shaw SR, Nihal M, Mukhtar H, Ahmad N. Sanguinarine causes cell cycle blockade and apoptosis of human prostate carcinoma cells via modulation of cyclin kinase inhibitor-cyclin-cyclin-dependent kinase machinery. Mol Cancer Ther. 2004 Aug;3(8):933-40.

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4. Ahsan H, Reagan-Shaw SR, Eggert DM, Afaq F, Mukhtar H and Ahmad N: Plant alkaloid sanguinarine prevents ultraviolet B radiation-mediated damages in the skin of SKH1 hairless mouse. J Invest Dermatol 126 (4; Suppl): 141, 2006.

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Conclusions:

In conclusion, we have made significant overall progress in this study funded by the 'US Army Medical Research and Material Command'. However, unfortunately, our progress during this reporting period (01/16/2006 - 01/15/2007) was hampered due to several unforeseen circumstances. Because of this reason, a one-year no-cost extension of the grant was obtained in January 2007. We are confident that our ongoing *in vivo* experiments with TRAMP mice will provide significant new information regarding the chemopreventive effects of sanguinarine against CaP. We will also evaluate the effect of sanguinarine on i) induction of apoptosis, ii) cellular proliferation, and iii) modulations in NF-κB-pathway, during sanguinarine-mediated inhibition of prostate tumorigenesis in TRAMP mice.

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